Invertase Inhibitor from Potatoes: Purification, Characterization, and Reactivity with Plant Invertases

By

Russell Pressey

Red River Valley Potato Processing Laboratory[1], East Grand Forks, Minnesota 56721

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Summary. Invertase inhibitor was extracted from potato tubers and purified nearly 1000-fold. The purification procedure involved precipitation at pH 4.0, fractionation with ammonium sulfate, adsorption on alumina C_γ gel, and gel filtration on Sephadex G-100 and DEAE-Sephadex A-50. The product obtained was homogeneous to electrophoresis on polyacrylamide gel. Exclusion chromatography on Sephadex G-100 indicated a molecular weight of about 17,000. The inhibitor did not inhibit yeast, *Neurospora,* and several plant invertases. It completely inhibited potato tuber invertase and a number of other plant invertases. Some plant invertases were partially inhibited.

Natural protein inhibitors have been reported for numerous enzymes, but most frequently for the hydrolases. Usually the inhibitor and the enzyme it inhibits occur together in a biological system, but some enzymes are known to be inhibited by proteins from diverse sources. For example, mammalian proteolytic enzymes are inhibited by proteins isolated from many plants. Trypsin inhibitors have been found in soybeans (7), potatoes (16), and alfalfa (12). Two chymotrypsin inhibitors have been crystallized from potato tubers (1, 13). A protein which inhibited both trypsin and pancreatic amylase was isolated from navy beans (2). The number of known inhibitors for glycoside hydrolases is not large, however. Kneen and Sandstedt (6) identified inhibitors for salivary and pancreatic amylases in wheat endosperm, rye, and sorghum. The amylase inhibitor from wheat flour was purified and its protein nature was established (9). A thermolabile inhibitor for pectinase has been detected in pear sap (18).

The first evidence for an invertase inhibitor in potato tubers was obtained from kinetic studies on invertase in crude extracts (15). The deviation from linearity between enzyme concentration and rate of invertase action was attributed to partial inhibition of invertase activity by an inhibitor in the extracts. The presence of an inhibitor was confirmed when it was found that it can be selectively inactivated by blending the crude extracts (10). This procedure was applied to study the levels of invertase and excess inhibitor in potato tubers as influenced by storage temperature (11). The results indicated an important role for the inhibitor in regulating invertase activity in the tubers.

The excess invertase inhibitor in warm stored tubers was partially purified earlier (10). The present paper describes the preparation and characterization of high purity inhibitor. Reactivity of the inhibitor with invertases from a variety of plants is described.

Materials and Methods

Purification of Potato Tuber Invertase. The invertase in extracts of potato tubers is always accompanied by the inhibitor. It is, therefore, necessary to inactivate the inhibitor before the invertase preparations can be used in the assay for the inhibitor. This can be accomplished by blending the extracts as was indicated earlier (10). The following modification of the original procedure for preparing partially purified invertase was employed in this study. Potato tubers (variety Kennebec) stored at 4° for several months were peeled and passed through a juicerator (Acme Manufacturing Co., Lemoyne, Penna.). Sodium sulfite was added to the juice to prevent enzymatic darkening. The juice was clarified by centrifugation and the supernatant solution was added to one-fourth volume of 1.0 M sodium acetate, pH 5.0. The sample was warmed to 37° and homogenized for 10 minutes in 100 ml portions with a VirTis homogenizer. The invertase was then purified by fractionation with ethanol. The precipitate obtained between 20 and 45 % ethanol, at −11°, contained most of the activity. It was collected and dissolved in a small volume of 0.2 M NaCl. Further purification was

[1] A laboratory cooperatively operated by the Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture; Minnesota Agricultural Experiment Station; North Dakota Agricultural Experiment Station; and the Red River Valley Potato Growers' Association.

achieved by filtration through Sephadex G-100. The alcohol fraction was applied to a 6 × 40 cm column previously equilibrated with 0.2 M NaCl which was also used for elution. The fractions containing the invertase were combined and stored at 4°. This procedure yielded invertase possessing a specific activity of approximately 150 with 20 % recovery. The assay procedure for invertase and the unit of activity were described earlier (10).

Assay for Inhibitor. A unit of inhibitor was defined earlier (10). The assay for inhibitor was performed with the partially purified invertase described above. Usually 3 levels of inhibitor were added to a constant amount of invertase and the residual activity was measured. A plot of residual activity versus the amount of inhibitor was linear over the range of 0 to 80 % inhibition. The amount of inhibitor in each sample was estimated from the corresponding plot. Protein was determined by the biuret method and absorption at 280 mμ (8).

Purification of Invertase Inhibitor. All steps were carried out at about 4° and centrifugations were conducted at 8500 × *g* for 10 minutes. Potato tubers (variety North Dakota 5899-1), stored continuously at 18° for 2 months after harvest, were washed, peeled, and passed through a juicerator. A ml of 0.8 M sodium sulfite was added to each 100 ml of extract to prevent enzymatic darkening. About 3 liters of extract were prepared in a typical purification procedure. The crude extract was clarified by centrifugation and a small portion was dialyzed against 0.2 M NaCl prior to analysis for the inhibitor. The pH of the extract was adjusted to 4.0 by addition of HCl and the precipitate that formed was collected by centrifugation. This precipitate was dispersed in 1 liter of 0.2 M NaCl and the pH was adjusted to 6.0. After stirring the suspension for 1 hour it was centrifuged and the sediment was discarded. The supernatant solution (acid fraction) was then fractionated with ammonium sulfate. The pH of the solution was adjusted to 5.5 and solid ammonium sulfate was added to give 25 % saturation. The precipitate was removed by centrifugation. The supernatant solution was adjusted to pH 4.0 and solid ammonium sulfate was added to give 35 % saturation. The precipitate was collected and dissolved in 400 ml of 0.2 M NaCl by adjusting the pH to 6.0. After dialysis of the ammonium sulfate fraction against 7 liters of 0.2 M NaCl, it was treated at pH 5.5 with 18 g of alumina Cγ gel (5 % solids). The alumina Cγ gel was collected by centrifugation, and the inhibitor was eluted with 75 ml of 0.2 M phosphate, pH 6.0, containing 0.5 M NaCl. This fraction (alumina Cγ gel I) was dialyzed against 0.2 M NaCl and then applied to a Sephadex G-100 column, 6 × 40 cm, previously equilibrated with 0.2 M NaCl. The column was eluted with 0.2 M NaCl at the rate of 75 ml/hour. The fractions containing high levels of inhibitor were combined (Sephadex G-100 fraction) and treated with 1 g of alumina Cγ gel. The inhibitor was eluted with 10 ml of 0.2 M phosphate, pH 6.0, containing 0.5 M NaCl (alumina Cγ gel II fraction), The eluate was dialyzed against 0.01 M phosphate, pH 6.0, containing 0.2 M NaCl. It was then applied to a DEAE-Sephadex A-50 column, 2 × 40 cm, previously equilibrated with the above solution. Elution was accomplished with this solution at the rate of 20 ml/hour. The fractions containing the inhibitor were combined (DEAE-Sephadex A-50 fraction) and treated with 0.6 g alumina Cγ gel. The inhibitor was eluted with 3 ml of 0.2 M phosphate, pH 6.0, containing 0.5 M NaCl (Alumina Cγ gel III fraction).

Studies with other Plant Invertases. Plants were collected at local farms and gardens during early summer. Extracts were prepared by blending 75 g of foliage with 75 ml of 0.2 M acetate buffer, pH 4.7, for 2 minutes. The macerates were strained through cheese cloth, centrifuged, and the supernatants were dialyzed against 0.2 M NaCl. The invertases were assayed according to the standard procedure.

Results

Purification of the Inhibitor. The selection of potato tubers for the isolation of invertase inhibitor is important because the level of excess inhibitor varies in the different varieties and also with storage temperature. A survey of the potato varieties grown in the Red River Valley area indicated that North Dakota 5899-1 contained the highest level of inhibitor. At harvest, this variety contained about 3 times more excess inhibitor than the variety Kennebec. High levels of excess inhibitor persisted in mature tubers stored continuously at 18°. In contrast, the inhibitor decreased to a low level in tubers stored at 4° as a result of an increase in the concentration of the enzyme invertase. The tubers stored at 18° remained in good condition for about 10 weeks and were generally used in the present study. However, it is possible to develop high levels of inhibitor in cold stored tubers by placing them at a warm temperature for several weeks.

A summary of the purification procedure is presented in table I. The acid precipitation step involved a considerable loss of inhibitor through incomp'ete precipitation. However, this step facilitated subsequent purification by removing most of the low molecular weight protein. The Sephadex G-100 step was one of the most important in the scheme. The pattern of separation is shown in figure 1.

The recovery of inhibitor was not high but the preparations obtained by this procedure were homogeneous according to gel electrophoresis (fig 2).

Properties of the Inhibitor. The inhibitor was stable over the pH range of 2 to 7 when incubated at 37° for 1 hour. At higher temperatures, maximum stability was observed between pH 3.5 and 4.5, with about 50 % loss of activity when heated to 61° for 5 minutes. The inhibitor was precipitated by trichloroacetic acid, but not by HCl, at pH 2.0. Digestion of the inhibitor with trypsin resulted in complete inactivation. Incubation of the inhibitor with 0.1 M

Table I. *Purification of Potato Tuber Invertase Inhibitor*

Fraction	Volume	Protein	Sp. act.	Yield
	ml	mg	units/mg protein	%
Crude extract	3000	33.000	30	...
Acid precipitate	1100	8300	75	62
Ammonium sulfate	400	4200	110	46
Alumina C_γ gel I	75	675	350	24
Sephadex G-100	175	57	2200	13
Alumina C_γ gel II	10	16	4500	7
DEAE-Sephadex A-50	77	2.7	21 000	6
Alumina C_γ gel III	3	1.2	29,000	3.5

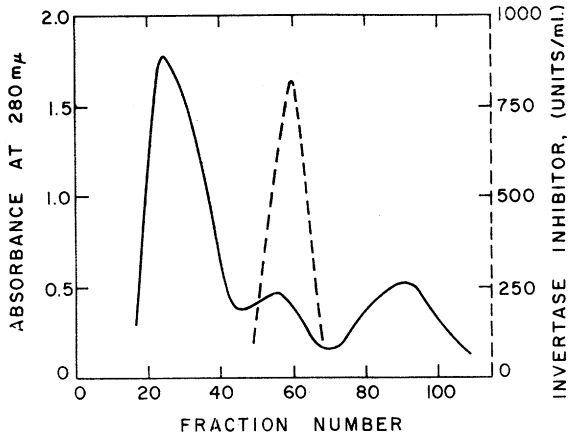

FIG. 1. Chromatography on Sephadex G-100 of the alumina C_γ gel I fraction. Protein concentration was determined by absorption at 280 mμ and the inhibitor was measured by the standard assay. Experimental conditions are described in the text. Each fraction contained 23 ml. ——— = Absorbance at 280 mμ. - - - - = Invertase inhibitor (units/ml).

13 ml

mercaptoethanol or cysteine produced slow but appreciable inactivation. The ultraviolet absorption spectrum of the inhibitor had a maximum at 280 mμ. The ratio of absorbance at 280:260 was 1.2. Tests for carbohydrate in the purified inhibitor were negative.

The estimation of the molecular weight was carried out on a Sephadex G-100 column, 2.5 × 90 cm, previously washed with 0.02 M phosphate, pH 6.0, containing 0.2 M NaCl. Three reference proteins were used, cytochrome c, egg albumin, and horse serum albumin, each at 10 mg/5 ml of the above buffer. Five ml of a Sephadex G-100 fraction inhibitor were used. The inhibitor and reference proteins were applied to the column separately and were eluted before application of the next sample. Elution was accomplished with 0 02 M phosphate, pH 6.0, containing 0.2 M NaCl. The reference proteins were assayed by absorption of 280 mμ and the inhibitor was assayed by the standard assay procedure. The elution volumes were as follows: cytochrome c, 311 ml; egg albumin, 217 ml, horse serum albumin, 183 ml, and invertase inhibitor, 289 ml. From these data, a

Table II. *Amino Acid Composition of Invertase Inhibitor*

Amino acid	% By weight of residue	Molar ratio methionine=1.0	Residues/17,000 mol wt
Asp	9.7	3.5	14
Thr	2.4	1.2	5
Ser	6.1	3.0	12
Glu	12.1	4.2	17
Pro	6.4	2.8	11
Gly	5.6	3.9	16
Ala	4.0	2.4	10
Val	6.5	2.8	11
Half-cyst	1.2	0.6	2
Met	2.8	1.0	4
Ileu	5.2	2.0	8
Leu	4.0	1.5	6
Tyr	5.7	1.6	6
Phe	4.8	1.5	6
Try	1.6	0.4	2
Lys	10.1	3.5	14
His	3.2	1.0	4
Arg	5.6	1.7	7

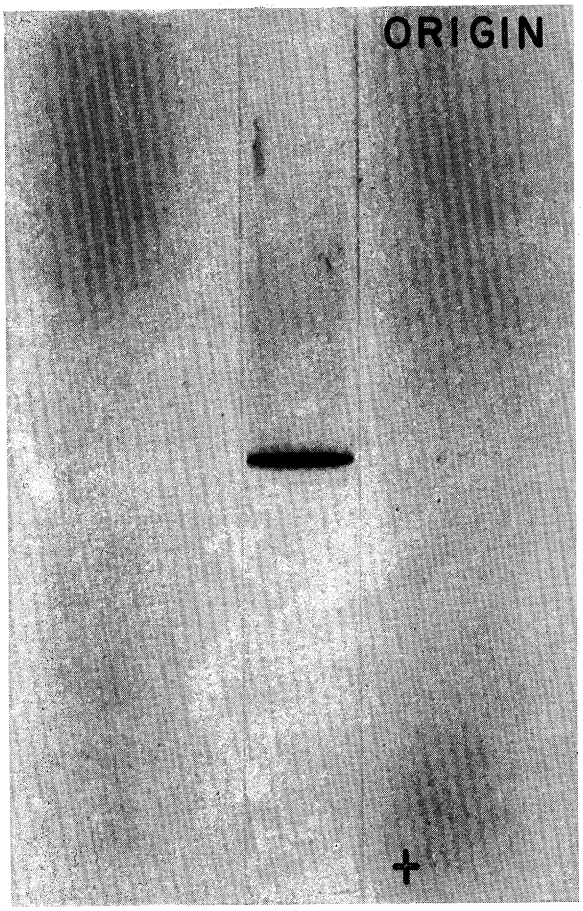

FIG. 2. Disc electrophoresis pattern of purified invertase inhibitor. Electrophoresis was conducted with a Canalco model 6 system (Canal Industrial Corporation, Bethesda, Maryland), using the standard polyacrylamide gel (7%, pH 9.5) at 5 ma for 1 hour. Forty μg of inhibitor was applied to the gel.

molecular weight of about 17,000 was estimated for the inhibitor (using molecular weights of 13,000 for cytochrome c, 45,000 for egg albumin, and 70,000 for horse serum albumin).

Amino Acid Analysis. Amino acid analysis was performed with a Technicon Auto Analyzer. The protein was hydrolyzed in 6 N HCl for 24 hours at 110° in sealed evacuated tubes. The amount of tryptophan relative to tyrosine was determined by the spectrophometric method of Goodwin and Morton (3). The amino acid composition is shown in table II. The values for serine, threonine and half-cysteine were not corrected for destruction during hydrolysis.

Effect of the Inhibitor on Potato Tuber Invertase. It was reported earlier that potato tuber invertase is noncompetitively inhibited by the inhibitor (10), and this was verified in the present study with purified inhibitor. Effectiveness of the inhibitor varied with pH (fig 3). Inhibition was most effective at about pH 4.5, but decreased as the pH was increased or decreased. At moderate levels of inhibitor, the invertase activity exhibited double pH optima with the larger optimum below pH 4.5. Invertase activity could be completely suppressed at all pH values, but

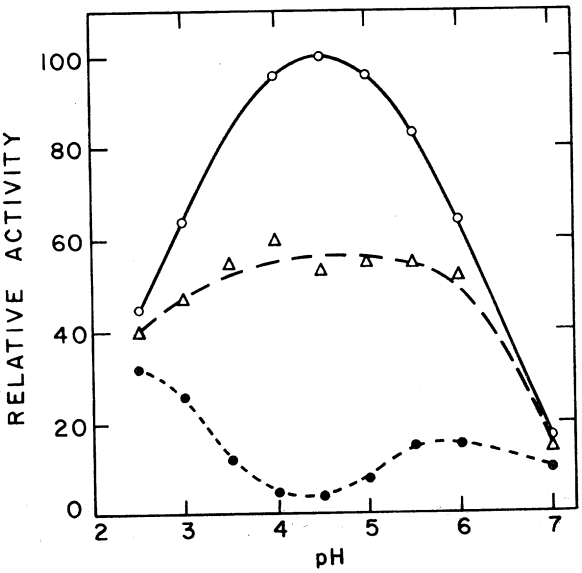

FIG. 3. Effect of invertase inhibitor on the pH optimum for potato tuber invertase. All the samples contained 2.0 units invertase/ml. ○—○, no inhibitor; △—△, 1.8 units inhibitor/ml; ●—●, 7.2 units inhibitor/ml. The buffers were prepared by adding 0.2 M dibasic phosphate to 0.1 M citric acid.

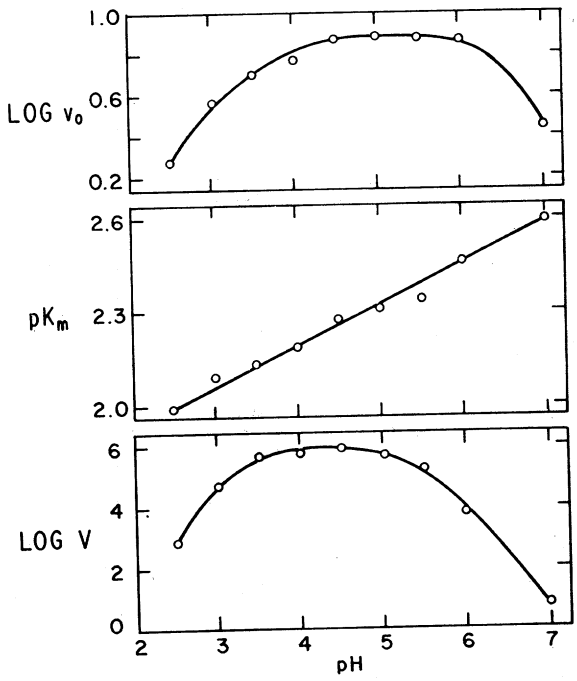

FIG. 4. Effect of pH on Km, V and v_o for potato tuber invertase acting on sucrose. The buffers were prepared by adding 0.2 M dibasic phosphate to 0.1 M citric acid.

the amount of inhibitor required increased in either direction from pH 4.5. The relationship between inhibitor concentration and percent inhibition, at a constant level of invertase, was linear at all pH values except as inhibition approached 100 %. At a constant level of inhibitor and increasing levels of invertase, the percent inhibition first decreased sharply and then gradually approached zero. This type of plot is consistent with an enzyme-inhibitor complex of low dissociability (5).

The unusual effect of pH on the inhibition of potato tuber invertase by the inhibitor prompted a re-examination of some of the kinetic properties of the enzyme. It was reported earlier that potato invertase exhibits a pH optimum at about 4.7 (10). A detailed study on the effect of pH on the activity revealed that the pH optimum is dependent on the substrate concentration. The pH optimum shifts toward the acid side when the substrate concentration is increased. The maximum velocity (V) and Michaelis constant (Km) were determined graphically at each pH from the velocity measurements at 5 sucrose concentration (1.2, 2.9, 5.8, 29, and 146 mM). The results are shown in figure 4. The Km values increased 4-fold as the pH was decreased from 7 to 2.5 and the pKm versus pH plot was linear. A broad maximum between pH 3.5 and 5.0 was obtained for log V, whereas the maximum for log v_o (v_o = V/Km) was obtained between pH 4.5 and 6.

Potato invertase, therefore, exhibits highest affinity for its substrate at about pH 6.0, which is the endogenous pH of the tubers. The pH optimum for the enzyme is also near 6.0 at low substrate concentrations. Because of its role in regulating invertase activity in the tuber, it is surprising that the inhibitor does not exert its greatest effect at pH 6.0 rather than at pH 4.5. The results of the kinetic studies on invertase do not provide an explanation for the double optima effect.

The Michaelis constants and relative maximum velocities for potato tuber invertase with the substrates sucrose, raffinose and stachyose at pH 4.5 are shown in table III. The relative amount of inhibitor required to inhibit 50 % of the reaction with each substrate and a constant level of invertase was also determined. Despite the great differences in V and Km for the 3 substrates, a given amount of inhibitor inhibits the reactions to the same extent.

Reactivity of the Inhibitor with Other Plant Invertases. The inhibitor did not inhibit yeast and *Neurospora* invertases. However, a study with higher

Table III. *Michaelis Constants and Relative Maximum Velocities for Potato Tuber Invertase Hydrolysis of Three Substrates*

Substrate	Michaelis constant	Relative max velocity	Relative amount of inhibitor to inhibit hydrolysis 50 %
	M × 10^3		
Sucrose	5.3	1.00	1.00
Raffinose	15	0.31	1.10
Stachyose	37	0.21	1.04

Table IV. *Effect of Potato Invertase Inhibitor on Invertases Isolated from Various Plants*

Scientific name	Common name	Invertase activity units/ml	pH optimum	Maximum inhibition	Units of inhibitor for half of max inhibition
				%	
Solanum tuberosum	potato (tuber)	26	4.5	100	1.0
" "	" (sprout)	102	4.5	44	0.9
" "	" (foliage)	80	4.5	57	1.1
Petunia hybrida	petunia	15	4.4	100	1.5
Nicotiana tabacum	tobacco	10	4.4	90	2.5
Lycopersicion esculentum	tomato	39	4.5	100	1.3
Hordeum sativum	barley	52	5.3	28	7
Bromus erectus	brome grass	26	5.0	7	...
Rheum rhaponticum	rhubarb	5	4.3	100	3.0
Medicago sativa	alfalfa	13	6.3	100	5.0
Betula pendula	weeping birch	5	3.5	0	...
Acer negundo	box elder	27	5.2	38	9
Melilotus alba	sweet clover	25	5.5	100	2.2
Armoracia lapathefolia	horse radish	96	5.4	89	2.7
Tulipa gesneriana	tulip	4	4.6	0	...
Helianthus annuus	sunflower	24	5.6	0	...
Asclepias syriaca	milkweed	10	5.8	100	1.4

plant invertases revealed that reactivity of the inhibitor is not limited to potato tuber invertase (table IV). Representative plants from a number of families were studied, but special emphasis was placed on the *Solanaceae*. The invertases were extracted from the foliage of the plants, unless indicated otherwise, and only the soluble invertases were tested. The invertase activity, pH optimum for the invertase, inhibition at pH 4.5 by the inhibitor, and the units of inhibitor required to produce half of the maximum inhibition were determined for each extract.

The reactivity of the inhibitor with the various plant invertases ranged from negligible to total inhibition. Invertases isolated from petunia, tomato, rhubarb, alfalfa and sweet clover foliage, as well as potato tubers, were completely inhibited at pH 4.5. Effectiveness of the inhibitor on these invertases, in general, was highest at about pH 4.5 and decreased as the pH was increased or decreased (fig 5). The invertases from tomato and petunia were especially resistant to inhibition at low pH, while the invertases from alfalfa and sweet clover were not inhibited at high pH. The residual activity for tomato invertase

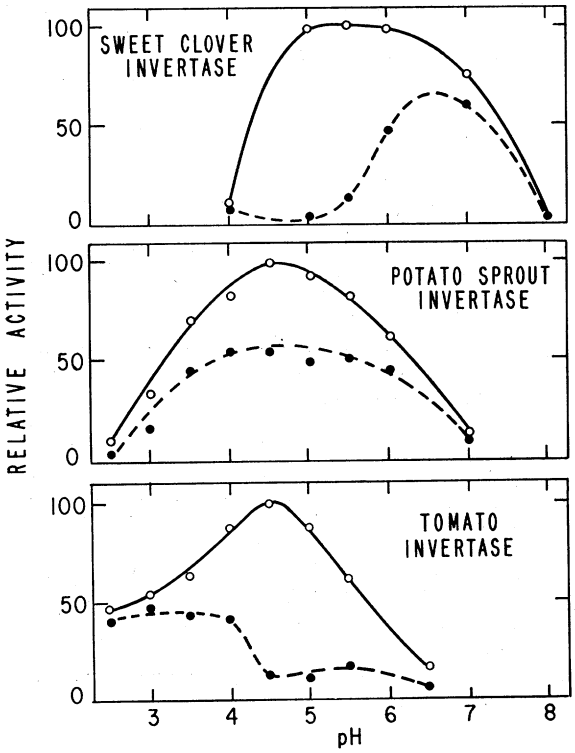

FIG. 5. Effect of invertase inhibitor on the pH optima for invertases isolated from sweet clover foliage, potato sprouts, and tomato foliage. The buffers were prepared by adding 0.2 M dibasic phosphate to 0.1 M citric acid. For each invertase, the open-circle plots were obtained in the absence of inhibitor. The closed-circle plots were obtained in the presence of 18 units inhibitor/ml for sweet clover invertase, 14 units of inhibitor/ml for potato sprout invertase, and 9 units inhibitor/ml for tomato foliage invertase.

in the presence of inhibitor possessed an apparent optimum at pH 3.5, and the residual activity for sweet clover invertase possessed an optimum at about pH 7.0 (fig 5).

Invertases from tobacco and horse radish foliage were also highly inhibited but residual activity was observed at pH 4.5 even in the presence of excessive levels of inhibitor. Invertases from potato sprouts, potato foliage, box elder, and barley leaves were only partially inhibited. The partial inhibition of potato foliage invertase is unusual because invertases from the foliage of other *Solanaceae* members were effectively inhibited. The inhibitor had little or no effect on invertases from brome grass, weeping birch, tulip, and sunflower foliage.

The amount of inhibitor required to maximally inhibit each invertase varied considerably with the source of the enzyme. Petunia, tomato, and potato sprout invertases were inhibited by levels of inhibitor comparable to that required for inhibition of potato tuber invertase. Sweet clover, rhubarb, horse radish, and tobacco invertases required moderate levels of inhibitor. Box elder invertase required the highest amount of inhibitor to partially inhibit its activity.

Discussion

The invertase inhibitor is a low molecular weight protein. Inactivation of the inhibitor by mercaptoethanol suggests that the molecule contains a disulfide bond which is necessary for activity. This is supported by the results on the amino acid composition of the purified inhibitor which indicate the presence of 1 cystine group per 17,000 molecular weight.

The mechanism of inhibitor action on invertase remains essentially unknown. Although direct evidence for an invertase-inhibitor complex was not obtained, it appears that an undissociable complex is formed. Reduction of invertase activity by increasing amounts of inhibitor occurs linearly, indicating that the effect of the inhibitor is on the free enzyme. Moreover, the same amount of inhibitor reduces the invertase activity to the same extent for 3 fructofuranoside substrates for which the Michaelis constants and maximum velocities differ greatly. The effect of the inhibitor on the invertase system, therefore, is different from the inhibition of invertases by aniline, which has been interpreted as a strong combination of aniline with the enzyme-fructose complex (17).

The inhibitor is effective for not only potato tuber invertase but also for many other plant invertases. This implies that invertases in other plants are similar to potato tuber invertase. Some of the invertases are obviously different on the basis of pH optima. However, there is the possibility that at least some of the invertase systems consist of several enzymes. For the systems that are inhibited by the inhibitor, inhibition is most effective at pH 4.0 to 5.0, indicating that maximum interaction of inhibitor and enzyme occurs in this pH range. Another explanation for

high residual activity at higher or lower pH is that these systems may contain several invertases with differences in reactivity with the inhibitor. For example, sweet clover invertase exhibits a very broad pH optimum (fig 5). The activity on the acid side of the optimum is completely inhibited by the inhibitor, leaving a large peak of residual activity at about pH 6.7. There is the possibility therefore that the invertase activity in extracts of sweet clover is due to 2 different enzymes. The evidence for 2 invertases, both with pH optima at pH 4.5, in both potato sprouts and foliage is more convincing. Because about half of the activity is inhibited by the inhibitor, it is reasonable to assume that half of the total activity is due to an invertase similar to potato tuber invertase. The remaining activity is not inhibited and must be attributed to another invertase.

There have been a number of reports on complex invertase systems in plants. Sacher (14) found both an intracellular, alkaline invertase (pH optimum, 8.0) and an outer space invertase (pH optimum, 4.0) in endocarp tissue of bean pods. Immature internodes of sugar cane contain an invertase with a pH optimum at about 5.2, but mature internodes contain a neutral invertase with a pH optimum at pH 7.0 (4). Moreover, it is well known that many plants contain insoluble, as well as soluble, invertases. The results of the present study demonstrate that plant invertases can be further differentiated on the basis of reactivity with potato invertase inhibitor. Some invertases, from a variety of plants, are similar to potato tuber invertase in terms of pH optima and reactivity with the inhibitor. However, because not all invertases are inhibited, it is possible to distinguish between 2 invertases possessing identical pH optima, as in extracts of potato sprouts and foliage.

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